

DESCRIPTION

FRUCTOSYLAMINE OXIDASE

5 TECHNICAL FIELD

The present invention relates to a novel fructosylamine oxidase, more particularly, to a fructosylamine oxidase derived from *Fusarium proliferatum*, a process for preparing the same, and use thereof in the measurement of amadori compounds.

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BACKGROUND ART

Amadori compounds are formed when a reactive substance having an amino group(s) such as protein, peptide or amino acid co-exists with a reducing sugar such as glucose in blood and food product.

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Thus, they combine together non-enzymatically and irreversibly through the amino group and aldehyde group, which is followed by amadori rearrangement to form an amadori compound. Since the production rate of an amadori compound is a function of concentration of reactive substances, contacting period, temperature, and the like,

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various information about a sample containing such reactive substances can be obtained from the amount of amadori compound. Therefore, analysis of amadori compounds is useful in the fields related to medicine, food, and the like. In the medical field, attention is particularly focused on the glycated protein as an index for diagnosis

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and control of conditions of diabetes. Diabetes causes various systemic symptoms (complications) such as diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, and the like, and is the leading cause of blindness and introduction of dialysis. These complications are linked not only to the restriction of daily life and

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social activity of patients but also to the swelling medical expenses and

raise a serious social problem. The importance of early detection and the following adequate control of blood glucose level has been indicated.

As an index for controlling blood glucose in diabetes, glycohemoglobin reflecting the mean glucose level for the past about 1
5 to 2 months, glycoalbumin reflecting the mean glucose level for the past about 2 weeks, or fructosamine corresponding to glycated protein having reducing ability in serum is measured. Glycohemoglobin (HbA1c) is a glycated hemoglobin wherein α -amino group of valine at N-terminus of hemoglobin β chain is glycated. The measurement of
10 HbA1c plays an important role in control of blood glucose level of diabetic patients.

The determination of amadori compound in enzymatic assay is carried out by contacting an amadori compound with an oxidoreductase, and measuring the amount of hydrogen peroxide produced or that of
15 oxygen consumed. Fructosylamino acid oxidase, one of oxidoreductases, has generally been purified from microorganisms. See, for example, JP-H06-65300B, JP-H03-155780A, JP-H07-289253A ([00319, [0037]), JP-H08-154672A (Claim 2 and [0027]), JP-H11-243950A ([0037]) and JP-H05-192193A.

20 Enzymes described in these publications are explained in brief below. Enzymes from *Corynebacterium* include those specific for an amino acid glycated at α -amino group but not active on fructosyl lysine (hereinafter, it may be referred to as "FL"), which are poorly heat stable (90% or more activity is decreased by treatment at 45°C for 10 minutes)
25 and hence lack in sufficient practical usefulness (JP-H06-65300B). Enzymes from *Aspergillus* include those less active on FL compared to fructosyl valine (hereinafter, it may be referred to as "FV"); however, it is unknown whether or not the enzyme is active on glycated protein or hydrolysates thereof (JP-H03-155780A). Enzymes from *Gibberella*
30 include those showing high specificity to fructosyl N α -Z-lysine

(hereinafter, it may be referred to as "FZL"), of which α -amino group is protected, and being active on fructosylpolylysine but not active on fructosyl valine (JP-H07-289253A, [0031] and [0037]). Enzymes from *Fusarium* include those having the same or higher activity for fructosyl lysine compared to fructosyl valine (JP-H08-154672A, Claim 2 and [0027]). Other enzymes from *Fusarium* or *Gibberella* include those inactive on fructosyl valine but specific for fructosyl lysine (JP-H11-243950A, [0037]).

However, these existing enzymes are not satisfactory in terms of, for example, activity in the determination of glycohemoglobin, and therefore there has been a demand for an enzyme with high activity and excellent specificity. For instance, although these existing enzymes are active on glycated amino acids or poly-lysines produced by fragmentation with protease treatment or the like, they are almost inactive on glycated peptides in which the α -position is glycated. Accordingly, in the case of glycohemoglobin, wherein α -amino group of N-terminal amino acid is glycated, it is necessarily to release the N-terminal fructosyl valine certainly beforehand.

To measure glycated proteins accurately using an existing fructosylamino acid oxidase, it is generally inevitable to surely release the glycated amino acid as a substrate of the enzyme. However, there have not been provided any methods by which the glycated amino acid of interest can be surely released or proteases which are highly specific enough to make it sure the same. One of strategies to solve this issue is to use a fructosylamine oxidase reactive on peptide itself which is glycated at N-terminus. It is particularly important to use a fructosylamine oxidase that is also active on glycated peptides as fragmentation products so that one can measure accurately the hemoglobin A1c (HbA1c) which is significant in control of diabetes.

DISCLOSURE OF INVENTION

The present invention provides a novel fructosylamine oxidase (hereinafter, it may be referred to as "FAO") useful in the accurate and efficient measurement of amadori compounds, specifically, glycated proteins.

The present inventors have intensively studied and found that a strain of *Fusarium* produces FAO with excellent substrate specificity and established the present invention.

Thus, the present invention provides s fructosylamine oxidase derived from *Fusarium proliferatum*.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows the elution pattern from Resource Q column chromatography of protein (OD = 280nm) and activity of cultured *Fusarium proliferatum*.

Fig. 2 is a graph showing the relationships between the activity of FAO-Q1 in a solvent and pH, which FAO-Q1 is one of enzymes of the present invention.

Fig. 3 is a graph showing the relationships between the activity of FAO-Q2 in solvent and pH, which FAO-Q2 is one of enzymes of the present invention.

Fig. 4 is a graph showing the relationships between the activity of FAO-Q1 in a solvent and temperature.

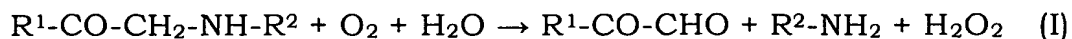
Fig. 5 is a graph showing the relationships between the activity of FAO-Q2 in a solvent and temperature.

Fig. 6 is a graph showing the molecular weights of FAO-Q1 and FAO-Q2 determined by gel filtration.

BEST MODE FOR CARRYING OUT THE INVENTION

The fructosylamine oxidase of the present invention has a

catalytic activity in a reaction shown by the scheme (I).



wherein R^1 is $-\text{[CH(OH)]}_n\text{-CH}_2\text{OH}$ (wherein, n is 5 or 6), and R^2 is an amino acid residue or a peptide residue consisting of 2 to 10 amino acids.

In the scheme (I) above, R^2 is an amino acid residue or a peptide residue consisting of 2 to 10 amino acids, preferably an amino acid residue or a peptide residue consisting of 2 to 6 amino acids, and more preferably an amino acid residue or a peptide residue consisting of 2 to 3 amino acids.

Amino acid(s) constituting R^2 varies depending on the amadori compound to be determined; however examples include valine, lysine, histidine, leucine, serine and the like. When R^2 is a peptide residue, it may consist of 2 to 10 amino acids with valine or leucine at the N-terminus. More preferred peptide may consist of 2 or 3 amino acids with valine at the N-terminus, and examples include valine-histidine and valine-histidine-leucine.

When the FAO of the present invention is used in the measurement of HbA1c, it is preferred that said FAO is active on valine glycated at α -amino group, i.e., fructosyl valine (FV) or a peptide having FV at the N terminus, as described above. On the other hand, when the FAO is used in the measurement of glycated albumin wherein ϵ -amino group of lysine is glycated, it is preferred that said FAO is active on lysine glycated at ϵ -amino group, such as fructosyl lysine (ϵ FL) or a peptide comprising ϵ FL.

The FAO of the present invention is not limited to the one derived from a particular origin as far as it has an enzyme activity. For example, an FAO which is produced by a microorganism growing in a medium containing a given glycated amino acid or glycated peptide as the sole carbon and nitrogen sources and showing enzyme activity on

glycated amino acid and glycated peptide as a substrate is useful for the present invention. Examples of glycated peptide used in the screening of such a microorganism include a product of fragmentation of an objective glycated protein. An objective FAO can be obtained by
5 culturing microorganisms in a medium containing such a glycated peptide as the sole carbon and nitrogen sources, purifying the resulting enzyme and confirming the activity. As will be described hereinafter, the inventors have screened microorganisms in soil using fructosyl valine-histidine-leucine (FVHL) and found out a microorganism of
10 *Fusarium* having an FVHL assimilating ability.

Since the FVHL above is the same as the N-terminal sequence of hemoglobin β -chain, it is suitable for screening of FAO useful in the measurement of HbA1c. Such a glycated peptide can be prepared according to a method known in the art.

15 Thus, the FAO of the present invention can be prepared microbiologically using a *Fusarium* strain. Preferred microorganisms include *Fusarium proliferatum* or variants thereof.

Fusarium proliferatum is a strain which the present inventors have isolated from soil for the first time according to the method
20 described in Example 1. It had been deposited with the "International Patent Organism Depository, National Institute of Bioscience and Human-Technology", Central-6, 1-1-1 Higashi, Tsukuba-shi, Ibaraki-ken, Japan (*Fusarium* sp. GL2-1 strain; received date: September 9, 2002; accession number: FERM P-19005), and has been transferred to
25 international deposit (transfer date: August 11, 2003; accession number: FERM BP-8451). Hereinafter, *Fusarium proliferatum* of the present invention may be referred to as "GL2-1" or "GL2-1 strain".

It is possible to derive strains having an improved activity on FVHL or other substrates from GL2-1, the original strain, by means of
30 mutagenesis or gene recombination techniques. Such a variant can

also serve as the source of FAO of the present invention. The derivative strains include those obtained artificially by mutagenesis or those obtained by screening.

The FAO of the present invention can be prepared by culturing
5 a microorganism capable of producing FAO in a glucose-valine browning medium (hereinafter, referred to as "GV browning medium"). The GV browning medium can be obtained by autoclaving glucose and valine at 120°C for 30 minutes. Examples of preferred GV browning medium includes a medium containing 1.5% glucose, 0.5% L-valine,
10 0.1% K_2HPO_4 , 0.1% NaH_2PO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$ and 0.2% yeast extract.

Typically, cultivation is performed at 25 - 37°C, preferably at 28°C. The pH of medium is between 4.0 and 8.0, preferably between 5.5 and 6.0. However, the conditions are not critical and should be
15 adjusted appropriately depending on the conditions of respective microorganisms and are not limited to the conditions described above.

When GL-2 strain is cultured under the above conditions for 12 - 36 hours, preferably for 24 hours, an FAO is accumulated in fungal cells. A cell-free extract can be obtained in a conventional manner by
20 collecting fungal cells by filtration followed by centrifugation. The grinding of cells can be carried out in a conventional manner, for example, by means of mechanical grinding, autodigestion using a solvent, freezing, ultrasonic treatment, pressurization, or the like.

The method of isolation and purification of an enzyme is also
25 known in the art. It can be conducted by combining appropriately known methods including salting-out with ammonium sulfate, precipitation with an organic solvent such as ethanol, ion-exchange chromatography, gel filtration, affinity chromatography, and the like.

For example, mycelia can be harvested from resultant culture
30 by centrifugation or suction filtration, washed, suspended in 0.1 M Tris-

HCl buffer (pH 8.0) containing 1mM DTT, ground (broken) with Mini-BeadBeater™ (0.5 mm glass beads), and centrifuged. The supernatant as a cell-free extract is then purified by ammonium sulfate fractionation, dialysis and column chromatography using Resource Q column (Amersham Biosciences).

Alternatively, when an FAO is secreted or accumulated in medium, the enzyme can be separated and purified according to a method known *per se*, for example, by an appropriate combination of methods including ion-exchange resin treatment, activated carbon absorption treatment, precipitation from organic solvent, vacuum concentration, freeze-drying, crystallization, and the like.

According to the method above, at least two FAOs have been obtained from GL2-1 strain, which FAOs show different retention times on Resource Q column chromatography. One of the FAOs is active on both of fructosyl valine (FV) and N- α fructosyl lysine (FZL) (hereinafter, referred to as "FAO-Q1"), and the other is active on FV but inactive on FZL (hereinafter, referred to as "FAO-Q2"). Although the preparation and identification is herein described in relation to FAO-Q1 and FAO-Q2 derived from GL2-1 strain, the present invention is not limited to an enzyme of particular origin and includes any FAOs which are useful for the purpose of the present invention and have the physicochemical characteristics shown below.

The enzymes of the present invention derived from GL2-1 strain will be hereinafter described in more detail.

FAO-Q1

- 1) It is almost equally or more active on fructosyl valine as compared to fructosyl lysine;
- 2) The optimum pH for enzyme reaction is 7.5;
- 3) The optimum temperature for stability of enzyme is about 30-40°C; and

4) The molecular weight is about 39 kDa when estimated by SDS-PAGE, and is about 39.4 kDa when estimated by gel filtration.

FAO-Q2

1) It is not detectably active on fructosyl lysine but is active on fructosyl valine;

2) The optimum pH for enzyme reaction is 7;

3) The optimum temperature for stability of enzyme is about 30-40°C; and

4) The molecular weight is about 49 kDa when estimated by SDS-PAGE, and is about 58 kDa when estimated by gel filtration.

General characteristics of these two types of enzymes are described below.

1. Normal Induction Characteristics

They are inducible enzymes that could be induced by FVHL, and are induced in a medium containing FVHL as the sole carbon and nitrogen sources.

2. Reaction Specificity and Substrate Specificity

As described in Example 2(1), enzymes partially purified from GL2-1 strain culture gave active fractions Q1 and Q2 of different retention times on Resource Q column chromatography. Each fraction contained an enzyme herein referred to as "FAO-Q1" and "FAO-Q2", respectively. As mentioned above, FAO-Q1 was almost equally active on both of FV and FZL, and on FVL as well. On the other hand, FAO-Q2 was active on FV as well as FVH and FVHL, of which N-terminal valine is glycated, but was inactive on FZL.

3. pH and Temperature Conditions

Determination of optimum pH

Enzyme reaction was conducted under different pH conditions between 3.5 and 10.0 according to the method for determination of activity as described above.

The buffers used were 100 mM acetate buffer at pH range of 3.5-6.0, 100 mM potassium phosphate buffer at pH range of 6.0-8.0, 100 mM Tris-HCl buffer (pH range of 7.0-9.0) and 100 mM glycine-NaOH buffer at pH range of 9.0-10.0. As shown in Figs. 2 and 3, it was revealed that the enzyme FAO-Q1 of the present invention has a pH optimum of about 7.5 at 30°C, and FAO-Q2 of the present invention a pH optimum of about 7.0 at 30°C.

Determination of optimum temperature for stability of enzyme

Temperature conditions for the enzyme were determined by incubating FAO-Q1 or FAO-Q2 in 0.1 M Tris-HCl buffer (pH 8.0) at a temperature between 30 and 65°C for 10 minutes, and measuring the activity under normal conditions. The results of measurement are shown in Figs. 4 and 5. These figures show that the optimum temperature for enzymatic stability is between 30°C and 40°C.

4. Evaluation of Titer

Titration of enzyme can be performed by a method known in the art (e.g., kinetic method), for example, that described in Example 1 (3). In this method, hydrogen peroxide generated by the reaction of an FAO with a glycosylated amino acid or peptide is measured on the basis of absorbance (505 nm) of quinone pigment produced in the presence of hydrogen peroxide. The amount (μmole) of hydrogen peroxide generated per minute is calculated on the basis of molar absorptivity ($5.16 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) of quinone pigment and the resultant numerical value is taken as a unit (U) of enzyme activity.

The method of measuring activity is not limited to the above-described method, and the enzyme activity of the present FAO can be determined by other methods including an end point method, a method based on measurement of oxygen absorption, etc.

Determination of Michaelis constant

Michaelis constant for respective substrates can be determined

by measuring the initial reaction rate in the process for determination of titer as described above while keeping the conditions regarding enzyme concentration, pH, temperature, and the like constant and changing only the concentration of substrate.

5 Among FAOs of the present invention, FAO-Q1 shows almost the same activity on FV and FZL and therefore is widely useful for assay of amadori compounds. On the other hand, FAO-Q2 shows activity on FV but not on FZL, and therefore is useful for a selective assay of glycohemoglobin. Furthermore, FAO-Q2 is active on FVH and FVHL
10 which are N-terminal sequence of glycohemoglobin. Accordingly, it becomes possible to determine only the glycosylation at N-terminus without measuring the internal glycosylation (ϵ -position) of glycohemoglobin molecule, whereby one can assay HbA1c more accurately.

15 When analyzing an amadori compound such as glycated protein using the present FAO, a sample containing an amadori compound(s) is contacted with an FAO of the present invention, and the amount of oxygen consumed or that of hydrogen peroxide generated is measured according to a known method. Any samples are available, and
20 examples include those derived from a living body such as blood (e.g. whole blood, plasma or serum) and urine, and food products such as soy sauce, and the like. Blood is an especially preferred sample.

 When an FAO of the present invention is used, the suitable reaction conditions such as pH and temperature are selected for
25 respective enzymes. That is, when FAO-Q1 is used, the reaction could be carried out at pH range of about 6.5-12, preferably about 7-8, more preferably about 7.5; and at temperature range of 30-40°C.

 When FAO-Q2 is used, the reaction could be carried out at pH
30 range of about 6-10, preferably about 6.5-8, more preferably about 7; and at temperature range of 30-40°C. However, the conditions may be

changed in accordance with the substrates or other reaction conditions etc., and are not limited thereto.

The amount of FAO used in an assay may be selected appropriately in accordance with the method used in the assay; however, it is generally 0.1 unit/ml or more, preferably 1-100 units/ml. As a buffer, Tris-HCl or the like can be used.

When analyzing a glycated protein using an FAO of the present invention, the protein is preferably subjected to fragmentation beforehand so that it releases an amino acid or peptide residue. Such methods including chemical and enzymic methods are known in the art.

However, since the FAO of the present invention, especially FAO-Q2, is active on not only glycated amino acid but also glycated peptide as the degradation products of glycated protein, measurement can be carried out with good accuracy even if fragmentation treatment is not perfect.

Accordingly, the present invention also provides a method of measuring amadori compounds in a sample using the above-described FAO (FAO-Q1 or FAO-Q2).

An FAO used in the measuring method of present invention can be prepared by culturing *Fusarium proliferatum* (FERM BP-8451) producing FAO in a nutrient medium, and isolating and purifying resulting FAO of the present invention from the medium. The so obtained FAO, namely a naturally occurring FAO, may have naturally occurring modifications and mutations as far as it meets the purpose of the present invention. Furthermore, it may be accompanied by contaminants, other than the enzyme, resulting from the isolation and purification steps subject that they do not affect the accuracy and reliability of the measurement.

The FAO of the present invention can also be prepared according to the recombinant DNA techniques. Namely, recombinant proteins corresponding to FAO-Q1 or FAO-Q2 can be prepared in a

conventional manner using a DNA encoding the amino acid sequence shown in SEQ ID NO: 4 or 6.

Thus, the present invention provides an FAO comprising the amino acid sequence shown in SEQ ID NO: 4 or 6.

5 As used herein, the term, "FAO (including FAO-Q1 and FAO Q2)" refers to, if not otherwise specified, both of an enzyme isolated from naturally occurring microorganisms and that obtained recombinantly.

10 The present invention also provides a DNA encoding FAO of the present invention.

The DNA of the present invention preferably encodes a protein comprising the amino acid sequence shown in SEQ ID NO: 4 or 6, and more preferably comprises the nucleotide sequence shown in SEQ ID NO: 3 or 5.

15 The process for preparing a recombinant protein by recombinant DNA technology is known in the art. For example, a recombinant protein having a desired activity can be prepared by introducing the DNA of the present invention into a suitable host, culturing the resultant transformant, and separating and purifying the FAO of the
20 present invention from the culture. As is easily understood by one of ordinary skilled in the art, the recombinant FAOs of the present invention obtainable in this manner are not limited to those having the amino acid sequences shown in SEQ ID Nos. 4 and 6, and rather encompass proteins having an amino acid sequence derived from the
25 said sequences according to a conventional manner and fragments of the amino acid sequences shown in SEQ ID Nos.4 and 6, as far as they fall within the definition above.

30 The preparation of recombinant FAO can be carried out according to a known method. For example, an expression vector for allowing expression of FAO in various hosts can be constructed by

inserting a DNA encoding FAO into downstream from a promoter of suitable expression vector. The expression vector is then used to transform a suitable host cell. Examples of host cell include microorganisms [prokaryotes (bacteria, such as *E. coli* and *Bacillus subtilis*) and eukaryotes (such as yeast)], animal cells or cultured plant cells. An appropriate host-vector system for each host is known and expression using such a host cell can be performed by a method described in literatures (e.g., Molecular Cloning: A LABORATORY MANUAL, Cold Spring Harbor Laboratory Press), or by a known method.

Transformation of host cells with an expression vector can also be performed by a method described in literatures (e.g., Molecular Cloning, *supra*), or by a method known in the art.

The cultivation of resultant transformants can be carried out in a suitable medium selected from known media or a medium freshly prepared. The medium usually contains a carbon source (e.g., glucose, methanol, galactose, fructose, etc.) and an inorganic or organic nitrogen source (e.g., ammonium sulfate, ammonium chloride, sodium nitrate, peptone, casamino acid, etc.). Other nutrients such as inorganic salts (e.g., sodium chloride, potassium chloride), vitamins (e.g., vitamin B1), and antibiotics (e.g., ampicillin, tetracycline, kanamycin) can be optionally added to the medium. For mammal cells, Eagle's medium is preferred.

The cultivation of transformants is normally conducted at pH 6.0-8.0, preferably at pH 7.0, and at a temperature of 25-40°C, preferably at 30-37°C for 8 to 48 hours. When the resulting FAO is present in the culture solution or filtrate thereof (supernatant), the cultured medium is filtered or centrifuged for separation. FAO can be purified from the filtrate/supernatant by a conventional method that is commonly used in the isolation and purification of a naturally occurring or a synthetic protein, which method includes dialysis, gel-filtration,

affinity column chromatography using anti-FAO monoclonal antibody, column chromatography using an appropriate adsorbent, high performance liquid chromatography, and the like. When the resultant FAO is present in the periplasm or cytoplasm of cultured transformants, cells are harvested by filtration or centrifugation, and subjected to ultrasonic treatment and/or lysozyme treatment for destruction of cell walls and/or cell membranes to obtain cell debris. The debris is then dissolved in an appropriate aqueous solution such as Tris-HCl buffer. FAO can be purified from the solution in accordance with the aforementioned method. If a fragment(s) having enzyme activity is needed, it can be obtained by treating the FAO with an enzyme such as restriction enzyme or exonuclease. Thus, FAO can be prepared efficiently by means of recombinant technology using appropriate host cells.

The following Examples further illustrate the present invention in detail.

Example 1: Screening and Identification of Microorganisms Producing FAO

(1) Screening of Microorganisms Producing FAO

Fructosyl valine-histidine-leucine (FVHL) which is the same as the N-terminal sequence of glycohemoglobin β chain was prepared by glycosylation of VHL. A method therefor is known to those skilled in the art.

FVHL-assimilating microorganism was isolated from soil using a medium (FVHL medium) containing FVHL as the sole carbon and nitrogen sources. Collected soil was added into 5 ml of FVHL medium in a test tube (16.5 mm in diameter), and cultured with shaking (300 rpm) at 30°C for 48 hours.

FVHL medium

FVHL

5g

	K_2HPO_4	1g
	NaH_2PO_4	1g
	$MgSO_4 \cdot 7H_2O$	0.5g
	$CaCl_2 \cdot 2H_2O$	0.1g
5	Vitamin mixture *	0.1% (v/v)
	Metal solution **	1.0% (v/v)
	<u>Distilled water</u>	<u>q.s.</u>
	Total volume	1,000 ml

*Vitamin mixture

10	Thiamine HCl	1 mg
	Riboflavin	2
	Calcium pantothenate	2
	Pyridoxine HCl	2
	Biotin	0.1
15	p-Aminobenzoic acid	1
	Nicotinic acid	2
	Folic acid	0.1
	<u>Distilled water</u>	<u>q.s.</u>
	Total volume	100 ml

20 ** Metal solution

	$MnSO_4 \cdot 3H_2O$	1.7 g
	$ZnSO_4 \cdot 7H_2O$	2.2
	$CuSO_4 \cdot 5H_2O$	0.4
	$CoCl_2 \cdot 2H_2O$	0.28
25	$Na_2MoO_4 \cdot 2H_2O$	0.26
	H_3BO_3	0.4
	KI	0.06
	<u>Distilled water</u>	<u>q.s.</u>
	Total volume	1,000 ml

30 As a result, thirteen FVHL-assimilating strains were obtained,

which were then subjected to cultivation and evaluation of activity as described below to select a microorganism strain(s) having FAO activity.

(2) Cultivation and Preparation of Cell-free Extract

Each of 13 strains obtained in (1) above was cultured in glucose-valine (GV) browning medium and the crude extract solution was prepared therefrom.

GV browning medium

Glucose	1.5 %(w/v)
L-valine	0.5
K ₂ HPO ₄	0.1
NaH ₂ PO ₄	0.1
MgSO ₄ · 7H ₂ O	0.05
CaCl ₂ · 2H ₂ O	0.01
Yeast extract	0.2

Cultivation was conducted by incubating in 5 ml of GV browning medium in a test tube (16.5 mm in diameter) with shaking (300 rpm) at 30°C for 24 hours. A cell-free extract was prepared by filtering the culture solution through a filter, grinding the resultant mycelia with Mini-BeadBeater™ (0.5 mm glass beads), and centrifuging (4°C, 10,000 x g, 10 minutes) the mixture, which was then used as a crude enzyme solution.

(3) Determination of FAO Activity

FAO activity of the crude enzyme solution was determined by the aforementioned rate method. The time-course of generation of hydrogen peroxide in the reaction mixture below was measured by a colorimetric method, and FAO activity was evaluated.

Tris-HCl buffer (pH 8.0)	100 µmol
4-Aminoantipyrine	4.5 µmol
Phenol	6 µmol
FV	5 µmol

Peroxidase	6 units
<u>Crude extract solution (cell-free extract)</u>	<u>1 ml</u>
Total amount	3 ml

A mixture (total 3 ml) except for the enzyme solution was
 5 equilibrated at 30°C. After adding the enzyme solution, the time-
 course of absorbance at 505 nm was measured. The amount (μmole) of
 hydrogen peroxide generated per minute was calculated on the basis of
 molar absorptivity ($5.16 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) of quinone pigment produced,
 and the resultant numerical value was taken as a unit(U) of enzyme
 10 activity. As a result, a strain having FAO was obtained.

(4) Identification of Strain

Mycological Properties

The microorganism was seeded on a plate of potato dextrose
 agar (PDA), oatmeal agar (OA) or 2 % malt agar medium (MEA), and
 15 cultured at 25°C up to 8 weeks while observing the mycological
 properties. Description of color of colony is in conformity with the
 teaching of Komerup & Wanscher (1978).

Observation of Macroscopic Characteristics of Colony

- The colony had a smooth edge and was slightly raised upward
 20 convexly.

- Aerial hypha was fluffy and the color of the colony surface was
 white-reddish white (11A1-2) from the beginning. After 8 weeks, it was
 not observed any apparent changes in the degree of color development
 or the color of surface due to conidia insertion.

25 - The color of colony on the backside was almost the same as
 that of the front side; slight pale red (11A3) color was observed in
 colonies cultured for a long time in PDA or MEA medium. Production
 of a small amount of clear exudate was observed in PDA or OA plate.

Observation of Microscopic Characteristics of Colony

30 - Both the microconidium and macroconidium were observed.

- Microconidium was of phialidic type and had a conidiophore structure like *Acremonium*. Conidiophore was almost straight and occasionally divided into two branches, and observed throughout the whole aerial mycelium. It was composed of one or two cells and sticky, and formed massive structure at the tip. The shape varied from ellipsoidal to fusiform shape and the surface varied from smooth to slightly rough surface.

- Macroconidium was morphologically the same as that of *Fusarium*, composed of 3 to 6 cells, was in the luniform shape, and had a smooth surface and footcell. There were observed many short aerial mycelia with a thickness of from middle to slightly thin. The cell wall was weak and most of macroconidia were deficient in the surface.

Considering the results above, the microorganism was assigned to *Fusarium* on the basis of the classification scheme described in Arx (1974), Domish (1993) and Malloch (1981). There are genera having a similar morphology such as *Cylindrocarpon*, *Candelabrella*, *Monacrosporium*, *Trichophoron* and the like; however, the microorganism of the present invention differs from these genera in, for example, that the macroconidium is in luniform shape, the aerial mycelium does not form a ring and there exists microconidium, and meets the definition of *Fusarium* described in "Gene of Hyphomycetes" (Carmichael et al., 1980).

This strain has been deposited with the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology under the accession number of FERM BP-8451 as "*Fusarium* sp. GL2-1 strain".

Identification of Species (Analysis of Ribosomal Base Sequence)

Identification of GL2-1 strain above was carried out by examining the sequence of 18S ribosome DNA (18SrDNA).

The GL2-1 strain was cultured in GV medium according to the

method described in (2) above and DNA was prepared in a conventional manner from the resultant mycelia. The internal transcribed spacer sequence of rDNA was then amplified by PCR using the resultant DNA as a template, and the base sequence was analyzed (Mycopathologia Vol. 140 P35-49 1997). As a result, the base sequence shown in SEQ ID NO: 1 was determined. The homology search with said base sequence revealed that it was 100 % homologous to *Fusarium proliferatum*.

Example 2: Preparation of FAO Using GL2-1 and Identification of the Same

(1) Partial Purification of FAO

1) Cultivation and preparation of cell-free extract

A GL-2 strain identified in Example 1 was cultured in 100 ml of GV browning medium as described in Example 1 (2) under the same medium composition and culture condition.

After cultivation, mycelia were collected by filtering the culture medium through a filter. The resultant mycelia (0.6 g) were suspended in 0.1 M Tris-HCl buffer (pH 8.0) containing 1 mM DTT, ground with Mini-BeadBeater™ (glass beads 0.5 mm), and centrifuged (4°C, 10,000 x g, 10 minutes) to obtain the supernatant as a cell-free extract.

2) Ammonium sulfate fractionation

Cell-free extract obtained in 1) was subjected to ammonium sulfate (30-80 % saturation) fractionation by dissolving in 50 mM-Tris-HCl buffer (pH 8.0) containing 1mM DTT and dialyzing against the same buffer.

3) Resource Q column chromatography

The ammonium sulfate fraction after dialysis was subjected to chromatography under the following conditions.

Analysis condition

Column (volume): Resource Q column (1 ml) (Amersham Biosciences K.K.)

Flow rate: 1 ml/ min
 Buffer A: 50 mM Tris-HCl buffer (pH 8.0) + 1 mM DTT
 Buffer B: Buffer A + 1M NaCl

Elution condition

5 0-5 min: 0 % Buffer B
 5-35 min: 0-5 % Buffer B
 35-40 min: 50-100 % Buffer B

The elution patterns of the protein (OD = 280 nm) and the activity on Resource Q column chromatography are shown in Fig. 1.

10 When FAO activity was monitored using FV as a substrate, two fractions (Q1 and Q2) were found to have activity. Measurement of activity was performed in a similar manner to that described in Example 1 (3). FAOs contained in these fractions are herein referred to as "FAO-Q1" and "FAO-Q2".

15 Table 1: Change of Activity according to Purification Steps

Step		total unit(U)	specific activity(U/mg)	yield(%)
Cell-free extract		0.5	0.019	100
After fractionation dialysis with 30-80% ammonium sulfate		0.3	0.0199	60
Resource Q	Q1	0.22	0.3	44
	Q2	0.03	0.067	6

(2) Comparison of Substrate Specificity of FAO-Q1 and FAO-Q2

The substrate specificity of the enzymes (FAO-Q1, FAO-Q2) contained in 2 fractions separated in (1), 3) above was determined. The
 20 FAO activity was measured using the respective two fractions as an enzyme solution according to the method described in Example 1(3). As a substrate, FV, FVH, FVHL, FVL, FVLS and FZL were used. The results are shown in Table 2.

Table 2: Substrate Specificity of FAO-Q1 and FAO-Q2

	Relative activity (%)	
	FAO-Q1	FAO-Q2
FV	100	100
FVH	n.d.	2.4
FVHL	n.d.	0.6
FVL	1.1	0.6
FVLS	n.d.	3.3
FZL	108	n.d.

FV: fructosyl valine; FVH: fructosyl valine-histidine; FVHL: fructosyl valine-histidine-leucine; FVL: fructosyl valine-leucine; FVLS: fructosyl valine-leucine-serine; FZL: fructosyl N- α -lysine

n.d.: not detected

It is clear from Table 2 that FAO-Q1 is almost equally active on both of FV and FZL, and on FVL as well, and that FAO-Q2 is active on FV but inactive on FZL, and is active on FVH and FVHL wherein N-terminal valine is glycosylated.

(3) Measurement of Km values

Km value (Michaelis constant) of FAO-Q1 or FAO-Q2 for FV or FZL were determined by measuring the activity according to the method described in Example 1(3) using as a substrate FV or FZL. The results are shown in Table 3.

Table 3: Km Values of FAO-Q1 and FAO-Q2 for FV or FZL

	FAO-Q1	FAO-Q2
FV	0.62	0.64
FZL	0.56	n.d.

n.d. not detected

FAO-Q1 and FAO-Q2 were comparable in Km value for FV. On the other hand, FAO-Q1 had smaller Km value for FZL than that for FV, indicating that said enzyme have a greater affinity for FV.

1) SDS electrophoresis

The molecular weight was determined by SDS electrophoresis using a gradient gel (gel concentration: 10-15 w/v %). The molecular weight of FAO-Q1 was about 39 kDa and that of FAO-Q2 was about 49

kDa, when measured using molecular weight markers (phosphorylase b: 97 kDa; bovine serum albumin: 68 kDa, ovalbumin: 45kDa, carbonic anhydrase: 32 kDa, trypsin inhibitor: 20.1 kDa, and α -lactalbumin: 14.4 kDa; Amersham Biosciences K.K.) as standard proteins of known molecular weight.

2) Gel filtration

The molecular weight was determined by gel filtration in a conventional manner using Superdex 200 (column size: 1 x 30 cm; Amersham Biosciences K.K.). The molecular weight of the enzymes of the present invention was calculated using a standard curve obtained from molecular weight markers (aldolase: 150 kDa, bovine serum albumin: 68 kDa, ovalbumin: 45 kDa, chymotrypsinogen A: 25 kDa, and cytochrome C: 12.5 kDa; Roche Diagnostics K.K.). The results are shown in Fig. 2. It was revealed that the molecular weight of FAO-Q1 is about 39.4 kDa and that of FAO-Q2 about 58 kDa.

(5) Analysis of Partial Amino Acid Sequence

In order to determine the N-terminal amino acid sequence, the purified FAO-Q2 enzyme was dialyzed against distilled water and 40 ng of the same was used as the sample for N-terminal amino acid sequencing. N-terminal 10 residues were analyzed using Protein Sequencer model 476A (Applied Biosystems, USA). The N-terminal sequence of FAO-Q2 was revealed to be the same as the amino acid sequence shown in SEQ ID NO: 2. On the other hand, it was impossible to determine the sequence of FAO-Q1 in this way because the N-terminus was blocked.

Example 3: Cloning of FAO cDNA

Genomic DNA of GL2-1 was prepared. FAO cDNA was then obtained by PCR using as a template the genomic DNA.

(1) Preparation of Genomic DNA of GL2-1 Strain

The genomic DNA was prepared from GL2-1 strain according to

a process comprising the following steps.

1. GL2-1 strain is liquid-cultured in 15 ml of DP medium (1% Dextone, 1% Peptone and 0.5 % NaCl, pH 7.4) at 30°C for 2 to 3 days.

5 2. Fungal cells (wet weight, 0.3 g) are collected by filtration through glass filter (3GL).

3. The resultant fungal cells are homogenized in a mortar containing liquid nitrogen with a pestle, further ground in a motor or the like, and then collected in a Corning tube.

10 4. After adding 2 ml of ice-cold TE buffer (10 mM Tris-HCl (pH8.0), 1mM EDTA), the mixture is vortexed lightly.

5. After adding 2 ml of a solution of 50 mM EDTA and 0.5 % SDS, the mixture is stirred by rotating several times and incubated at 37°C for 30 minutes.

6. The mixture is centrifuged (3,000 rpm, 10 minutes).

15 7. The supernatant is treated with phenol-chloroform (3 times) wherein stirring is conducted by rotation.

8. After adding 2.5 volumes of ethanol, the mixture is stirred by rotating several times. At this stage, filamentous DNA appears.

20 9. The mixture is briefly centrifuged (3,000 rpm, 5 minutes) to sediment filamentous DNA. When DNA does not become filamentous, the mixture is centrifuged according to the normal ethanol precipitation.

25 10. The precipitates are dissolved in 400 µl of TE buffer (10 mM Tris-HCl (pH8.0), 1mM EDTA; hereinafter, "TE buffer" has the same meaning), and transferred into an Eppendorf tube. After adding 5 µl of RNase (10 mg/ml), it is incubated at 37°C for 30 minutes.

11. After treating two times with phenol-chloroform, 2.5 volume of ethanol is added to the tube, which is followed by stirring thoroughly by rotation.

30 12. The filamentous DNA is transferred to a new tube with toothpick (excess ethanol is removed).

13. DNA is dissolved in 50-100 µl of TE buffer (pipetting gently but not vortex).

14. DNA is quantitatively determined. DNA (1 µg) was electrophoresed on agarose gel to confirm a band(s).

5 (2) Preparation of cDNA by PCR

1) Preparation of partial sequence (about 200 bp fragment)

A search for a region with high homology was carried out using an already-known total amino acid sequence of an FAOD from filamentous fungus. The following primers were designed on the basis of resulting information.

Primers:

Forward primer: 5'-GGBTTYTTCWTSGARCCNRAYGA-3' SEQ ID NO: 7

Reverse primer: 5'-GTRCVGYRYMCCAGCAVAT-3' SEQ ID NO: 8

15 PCR was performed using the above genome DNA as a template in a reaction solution of standard composition using Taq polymerase (TaKaRa Ex Taq, TAKARA BIO INC.).

PCR condition:

20	Primer (SEQ ID NO: 7)	0.2 µM
	Primer (SEQ ID NO: 8)	0.2 µM
	10 ExTaq PCR buffer (TAKARA BIO INC.)	10 µl
	Magnesium Chloride	2.5 mM
	Taq Polymerase(TAKARA BIO INC.)	2.5 U
25	D.D.W. (double deionized water) was added to make the total volume 100 µl.	

One cycle of 94°C for 1 minute; 35 cycles of (94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute); and 1 cycle of 72°C for 3 minutes.

30 After completion of PCR, 10 µl of reaction solution was electrophoresed on agarose gel and a band assumed to be the objective

fragment was observed at 200 bp. The band was excised, treated with TOPO TA Cloning Kit (Invitrogen) according to the instructions attached to the kit, and transformed into *E. coli* JM109. Twenty transformants were selected arbitrarily, and subjected to extraction of plasmid. Each plasmid was treated with restriction enzyme, and a plasmid(s) containing a DNA of appropriate size was selected and sequenced. The sequencing was performed using BigDye Terminator Cycle Sequence Kit and, as a sequencer, ABI PRISM3100 Genetic Analyzer.

As a result, two base sequences (polynucleotides) possibly corresponding to two isozymes (FAO-Q1 and FAO-Q2) were obtained. In the putative amino acid sequence deduced from the thus determined base sequence, the amino acid sequence of purified enzyme was confirmed. It was revealed that the DNA fragments amplified by PCR above contain a portion of genes each encoding FAO-Q1 and FAO-Q2, respectively.

2) Preparation of upstream or downstream partial sequences and total DNA

DNA sequences of upstream and downstream regions were determined from the two 200 bp fragments obtained in 1) above using TaKaRa LA PCR in vitro Cloning Kit. The resultant base sequences of FAO-Q1 and FAO-Q2 are shown in SEQ ID NO: 3 and SEQ ID NO: 5, respectively. The deduced amino acid sequences encoded thereby are shown in SEQ ID NO: 4 and SEQ ID NO: 6, respectively.

INDUSTRIAL APPLICABILITY

The present invention provides novel FAOs, which are expected to contribute to development of analysis of amadori compounds. In particular, using, among the present FAOs, an enzyme having activity on glycated peptide as well as glycated amino acid makes it possible to measure a glycated protein more accurately even if fragmentation of

glycated protein is not perfect. As a result, HbA1c which is important for control of glucose level in blood in diabetic patients can be determined accurately, and thereby contributing to the treatment of diabetes and the prevention of complications in diabetic patients.

- 5 Furthermore, the DNA encoding the novel fructosylamine oxidase of the present invention is expected to enable the efficient large-scale production of the enzyme by means of gene recombinant techniques, and thereby accelerating the development of analysis of amadori compounds.